FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 522-1784

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR 1.5): 10/089994

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/BE00/00120	October 6, 2000	October 6, 1999

TITLE OF INVENTION: **Isolation of Precursor Cells and Their Use For Tissue Repair**

APPLICANT(S) FOR DO/EO/US: **Frank Luyten, Cosimo DeBari and Francesco Dell'Accio**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31)
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   - a ☐ is attached hereto (required only if not communicated by the International Bureau)
   - b ☒ has been communicated by the International Bureau.
   - c ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2))
   - a ☐ is attached hereto
   - b ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   - a ☐ are attached hereto (required only if not communicated by the International Bureau)
   - b. ☒ have been communicated by the International Bureau
   - c ☐ have not been made, however, the time limit for making such amendments has NOT expired
   - d ☐ have not been made and will not be made
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4))
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5))
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. ☐ A copy of the International Search Report (PCT/ISA/210)

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
15. ☒ A **FIRST** preliminary amendment
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment
17. ☐ A substitute specification
18. ☐ A change of power of attorney and/or address letter
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4)
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information

Certified copies of priority documents.

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/089994	PCT/BE00/00120	522-1784

24. The following fees are submitted

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1040.00**	
☒ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$890.00**	
☐ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but international search fee (37 CFR 1 445(a)(2)) paid to USPTO	**$740.00**	
☐ International preliminary examination fee (37 CFR 1 482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$710.00**	
☐ International preliminary examination fee (37 CFR 1 482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1 492 (e)).		**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	21 - 20 =	1	x $18 00	**$18.00**
Independent claims	4 - 3 =	1	x $84 00	**$84.00**
Multiple Dependent Claims **(check if applicable)**			☐	**$0.00**
TOTAL OF ABOVE CALCULATIONS =				**$992.00**
☒ Applicant claims small entity status See 37 CFR 1 27) The fees indicated above are reduced by 1/2				**$496.00**
SUBTOTAL =				**$496.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**
TOTAL NATIONAL FEE =				**$496.00**
Fee for recording the enclosed assignment (37 CFR 1 21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3 31) **(check if applicable).** ☐				**$0.00**
TOTAL FEES ENCLOSED =				**$496.00**
			Amount to be: refunded	$
			charged	$

a. ☒ A check in the amount of **$496.00** to cover the above fees is enclosed

b. ☐ Please charge my Deposit Account No _____ in the amount of _____ to cover the above fees A duplicate copy of this sheet is enclosed

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No **12-0913** A duplicate copy of this sheet is enclosed

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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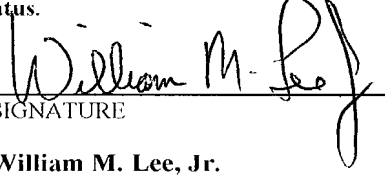

SIGNATURE

William M. Lee, Jr.

NAME

26,935

REGISTRATION NUMBER

April 2, 2002

DATE

522-1784

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE THE APPLICATION OF	)
	) Examiner:
Frank Luyten et al.	)
	) Group Art Unit No.
SERIAL NO.: To Be Assigned	)
	)
FILED: Herewith	)
	)
FOR: Isolation of Precursor Cells and Their Use For Tissue Repair	)

AMENDMENT ACCOMPANYING APPLICATION

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

The present application is the national filing of international application number PCT/BE00/00120. Appended hereto is a copy of the International Preliminary Examination Report for that application, having appended thereto the claims as they currently appear in the international application. Before calculation of the national filing fee for the United States, it is requested that the application be amended as follows:

IN THE CLAIMS:

Cancel claims 1 through 30 without prejudice and substitute new claims 31 through 51 as follows:

31. A method of positively identifying viable, expanded or passaged, committed, pluripotent skeletal precursor cells that have entered a post-natal differentiation pathway leading to skeletal

or connective tissues comprising the steps of

isolating mammalian cells into a cell culture in vitro, and

detecting the presence of a positive embryonic marker of an expressed bone morphogenic or cartilage derived morphogenic protein, a homolog thereof or a marker co-expressed and/or co-detectable with this marker.

32. The method according to claim 31, wherein the presence of the positive marker is further characterised by the absence of a negative marker, said negative marker preferably being FGFR3 or a marker or factor co-expressed or co-detectable with this negative marker.

33. The method according to claim 31 wherein the positive marker is an actively expressing gene, a protein or an mRNA expressed by a gene in the precursor cells or a part thereof, detectable at the DNA, mRNA, cDNA or the protein level and/or detectable via the activity of a promoter directing/regulating this gene expression, operably linked to a heterologous reporter gene.

34. The method according to claim 31 wherein the positive marker identifies precursor cells of a joint interzone in mammals.

35. The method according to claim 31 wherein the expressed bone morphogenic or cartilage derived morphogenic protein is the cartilage-derived morphogenic protein CDMP-1 or a transforming growth factor b having at least 80% homology with CDMP-1 as a marker of skeletal precursor cells from any part of the body or a marker or factor co-expressed or co-detectable with any or all of these positive markers.

36. A method according to claim 31, wherein the step of detecting the presence of the positive marker includes applying a binding agent for the positive marker to an isolated source of cell having the precursor cells, the marker positively identifying the cell and

separating the cells which are bound to the binding agent.

37. A method for sorting and/or enriching precursor cells in cell culture in vitro comprising selecting cells with reagents, ligands, and/or monoclonal or polyclonal antibodies recognising cell surface markers wherein the cell surface marker is co-expressed or co-detectable with the marker of claim 31, said precursor cells optionally being skeletal precursor cells.

38. A method for producing or repairing connective tissue into a mammal comprising administering skeletal precursor cells marked according to claim 31, said cells optionally being cultured at a cell density of at least 10^5 cells/ml and/or having a factor administered that stimulates differentiation of the skeletal precursor cells into the type of connective tissue to be produced or repaired.

39. A method of producing matrix comprising cultivating precursor cells marked according to claim 31 as matrix producing cells, said matrix optionally further comprising a bioresorbable polylmer or carrier.

40. A method for treating subglottic stenosis, tracheomalacia, chondromalacia patellae, osteoarthritis and traumatic lesions in a mammal said method comprising supplying precursor cells being marked according to claim 31.

41. A method for joint surface defect repair in a mammal comprising the co-implantation of chondrocytes and skeletal precursor cells marked according to claim 31.

42. A method for enhancing the implantation of a prosthetic device in connective tissue comprising the step of implanting a prosthetic device having skeletal precursor cells according to claim 31 adhered thereto under conditions suitable for differentiating the cells into the connective tissue desired. method of treatment.

43. A culture of isolated and expanded viable, differentiated, pluripotent precursor cells that have entered a post-natal skeletal differentiation pathway leading to skeletal or connective tissue, wherein the cells express a positive embryonic marker which is an expressed bone morphogenic or cartilage derived morphogenic protein, a homolog thereof or a marker co-expressed and/or co-detectable with this marker.

44. A therapeutic composition comprising the cells of claim 43.

45. An implant comprising the cells of claim 43, said implant being optionally suitable for connective tissue implantation.

46. A method of treating a patient in need thereof comprising administration of the therapeutic composition comprising the cells of claim 43.

47. A diagnostic for positively identifying in vitro a positive marker of viable, committed,

pluripotent skeletal precursor cells that have entered a post natal differentiation pathway leading to skeletal or connective tissues , wherein the marker is an expressed bone morphogenic or cartilage derived morphogenic protein, a homolog thereof or a marker co-expressed and/or co-detectable with this marker.

48. The diagnostic according to claim 47 wherein the diagnostic also identifies the absence of a negative marker.

49. The diagnostic according to claim 47 wherein the positive marker identifies precursor cells of a joint interzone in mammals

50. The diagnostic according to claim 47 wherein the expressed bone morphogenic or cartilage derived morphogenic protein is the cartilage derived morphogenic protein CDMP-1 or a transforming growth factor beta having at least 80 % homology with CDMP1 as a marker of skeletal precursor cells from any part of the body or a marker or factor co-expressed or co-detectable with any or all of these positive markers.

51. A method to positively identify viable committed skeletal pluripotent precursor cells that have entered a post natal differentiation pathway leading to connective or skeletal tissues, comprising selecting or identifying cells expressing an embryonic marker wherein the embryonic marker is an expressed bone morphogenic or cartilage derived morphogenic protein, a homolog thereof or a marker co-expressed and or co-dectable with this marker.

Remarks

The above amendments are being made in order to eliminate all claims from the international application to delete multiple dependency and improper multiple dependency, and replace the claims with a new claim set comprising claims 31 through 51. Should any claims remain in the international application except for the new claims above, that is unintended and the Patent and Trademark Office is requested to cancel any remaining claims in the international application before calculation of the national filing fee for the United States.

Examination of the application on its merits is awaited.

April 2, 2002

Respectfully submitted,

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ISOLATION OF PRECURSOR CELLS AND THEIR USE FOR TISSUE REPAIR.

The present invention relates to the field of tissue engineering in general, and more specifically to the identification of skeletal precursor cell populations for the repair of connective tissues, including skeletal tissues *in vivo*.

BACKGROUND OF THE INVENTION

Cartilage is a tissue composed by a cellular component, chondrocytes, and by an extracellular matrix typically rich in type II collagen and highly sulfated proteoglycans. The latter property confers cartilage its peculiar histochemical characteristics that are strong staining with alcian blue at low pH (0.2-2.5) and metachromasia with toluidine blue and safranin O. The abundance of type II collagen, link protein, and proteoglycan aggrecan, together with the presence of minor collagens such as type IX and type XI collagen, are hallmarks of cartilage tissue.In post-natal mammals cartilage contributes to the structure of several organs and systems like the articular surface of diarthrodial joints and other joint-associated structures such as menisci, the ear, the nose, the larynx, the trachea, the bronchi, structures of the heart valves, part of the costae, synchondroses, entheses etc. In some of the mentioned locations (e.g. entheses, the annulus fibrosus of the intervertebral disks, the menisci, insertion of ligaments etc.) for the abundance of collagens (mostly type I collagen) it is called *fibrocartilage*. In other locations (e.g. the pinna of the ear, epiglottis etc.) it is particularly rich of elastin and is called *elastic cartilage*. In all the other structures including articular cartilage, for its semi-transparent, clear aspect it is called *hyaline cartilage*.

During embryonic development of long bones, mesenchymal cells aggregate and differentiate to form cartilage anlagen, which provide the mold of the future long bones. These cartilage templates in development evolve undergoing endochondral bone formation through a cascade of events including chondrocyte hypertrophy, vascular invasion, mineralization, and eventually replacement by bone, except for a thin layer at the extremities of the bone elements that will differentiate into the articular surface of dyarthrodial joints. In this locations cartilage tissue remains hyaline for all the life-span of the individual. With aging, articular cartilage is well known to undergo a process of senescence, being affected in its mechanical properties and intrinsic resilience.

The current therapy for loss of cartilage tissue is replacement with a prosthetic material such as silicone for cosmetic repairs, or metal alloys for joint refinement. Placement of prosthetic devices, however, is a very artificial way of repairing, usually

associated with loss of underlying bone without recovery of the full function allowed by the original cartilage tissue. Serious long-term complications associated with the presence of a permanent foreign body can include infection, erosion and instability. Implantation of sterilized bone or bone powder with surgical steel seeded with bone cells has been largely unsuccessful because of the non-degradable nature of the cell support. U.S. Pat. No. 4,609,551 discloses that fibroblasts exposed *in vitro* for at least three days to a soluble bone protein are capable of stimulating a chondrogenic response *in vitro* and/or *in vivo*. The activated fibroblasts are then transferred *in vivo* by combining them with a biodegradable matrix, or by intra-articular injection or attachment to allografts and prosthetic devices. The disadvantage of this method is that chondrogenesis is not allowed to develop in the short-term cultures and there is an unduly heavy reliance on the exposed fibroblasts at the implant site for cartilage synthesis. EP-A-739,631 discloses producing a biological material comprising reconstituted cartilage tissue by growing chondrocytes on a flexible sheet of 1.5 mm thick demineralized natural bone. This, however, will be useful only when the bone is not self-derived, because harvesting self-derived bone requires a complicated and painful surgery.

Joint surface defects can be the result of various etiologies such as inflammatory processes, neoplasias, post-traumatic and degenerative events, etc. Whatever the cause, due to its limited capacity for repair, cartilage heals poorly with, at best, some scar formation or fibrocartilaginous tissue. This partial repair of the articular surface leads to osteoarthritis and severe functional disability. Based on the depth of the injury, two types of joint surface defects are defined, the osteochondral (or full-thickness) and the superficial (or partial-thickness).

Osteochondral (or full-thickness) joint surface defects include damage to the articular cartilage, the underlying subchondral bone tissue, and the calcified layer of cartilage located between the articular cartilage and the subchondral bone. They typically arise during severe trauma of the joint or during the late stages of degenerative joint diseases, e.g. during osteoarthritis. Since the subchondral bone tissue is both innervated and vascularized, damage to this tissue may be painful. Osteochondral defects rely on the extrinsic mechanism for repair. Extrinsic healing relies on mesenchymal elements from subchondral bone or joint-associated tissues to participate in the formation of new connective tissue, including skeletal tissue. This repair tissue may undergo metaplastic changes to form fibrocartilage that does however not display the same biochemical composition or mechanical properties as normal articular cartilage or subchondral bone and degenerates with use.

Superficial or partial-thickness injuries of the articular cartilage that do not

penetrate the subchondral bone rely on the intrinsic mechanism for repair. Soon after superficial injury, chondrocytes adjacent to the injured surfaces show a brief burst of mitotic activity associated with an increase in metabolic activity and matrix synthesis. Despite these attempts at repair, there is no appreciable increase in the bulk of cartilage matrix and the repair process is rarely effective in healing the defects. Although initially sometimes painless, partial-thickness defects often degenerate into osteoarthritis of the involved joint.

Repair of articular cartilage defects with suspensions of chondrocytes has been carried out in a variety of animal models and is now employed in humans (Brittberg M. et al., *N. Eng. J. Med.* 1994, 331:889-95). Autologous chondrocytes obtained from an unaffected area of the joint are released, expanded *in vitro* in the presence of autologous serum and subsequently injected under a periosteal flap sutured to cover the cartilage defect. This procedure has led to a proven at least symptomatic amelioration. This promising approach has still wide margins for improvement, since it is known that *in vitro* expansion of chondrocytes results, after a limited number of cell divisions, in a loss of their phenotypic stability (as defined by the ability of chondrocytes to form hyaline cartilage *in vitro* but also *in vivo*) making the cell suspension to be injected unreliable.

Three alternative approaches have been developed in an attempt to improve the success rate in treating mammalian articular cartilage defects. In the first approach, synthetic carrier matrices are impregnated with chondrocytes and then implanted into the cartilage defect where they hopefully produce and secrete components of the extracellular matrix to form articular cartilage at the site of the defect. A variety of synthetic carrier matrices have been used to date and include three-dimensional collagen gels (e.g. U.S. Pat. No. 4,846,835), reconstituted fibrin-thrombin gels (e.g. U.S. Pat. Nos. 4,642,120; 5,053,050 and 4,904,259), synthetic polymer matrices containing polyanhydride, polyorthoester, polyglycolic acid and copolymers thereof (U.S. Pat. No. 5,041,138), and hyaluronic acid-based polymers. Once a mitotically expanded population of chondrocytes is obtained, the cells can be implanted either back into the same subject from which their parental cells were originally derived (autologous implantation), or into a different subject (heterologous implantation). In addition, heterologous implantation may use chondrocytes obtained from a related or unrelated individual of the same species (allogeneic), or from a different species (xenogeneic). Alternatively, chondrocytes may be obtained from an established, long-term cell line that is either allogeneic or xenogeneic.

Autologous implantation requires that chondrocytes are harvested from an uninvolved area of the joint surface from the patient and then *in vitro* culture expanded

to sufficient number or density for an effective implant. The amount of time required for such sufficient expansion, however, may preclude the effective use of an autologous culture since some cartilage repairs should be carried out immediately or within a short time after a traumatic injury occurs. Another limitation is the mitotic potential of the cells, since there is a limitation to the number of times the cells can be expanded, and the ultimate quantity of cells produced for therapy may be limited. In addition, where a severe debilitating joint disorder causes general degradation of cartilage tissue throughout a patient's body, namely e.g. in elderly people, there may be very little unaffected cartilage tissue available from which to initiate a chondrocyte culture. The introduction of heterologous chondrocytes into a patient, on the other hand, may stimulate an undesirable immune response directed against the implanted material, leading to potential rejection of the newly formed and engrafted cartilage tissue. In addition, heterologous implantation risks the transmission to the subject of infectious agent(s) present in the tissue or cell line.

Moreover, when using synthetic carrier matrices neo-cartilage may be formed around the periphery of the implant thereby preventing integration of the implant into the cartilage defect. Monitoring the formation and development of the resulting synthetic cartilage *in situ* is difficult to perform and usually involves an arthroscopic or open joint examination. Furthermore, implants containing synthetic polymer components may be unsuitable for repairing large cartilage defects since polymer hydrolysis *in situ* inhibits the formation of cartilage and/or its integration into the defect.

In the second approach, the defect is filled with a biocompatible, biodegradable matrix containing chemotactic and mitogenic growth factors to stimulate the influx of chondrocyte progenitor cells into the matrix *in situ*. The matrices optimally contain pores of sufficient dimensions to permit the influx into, and proliferation of the chondrocyte progenitors within the matrix. The matrix also may contain growth factors to stimulate the differentiation of chondrocyte progenitor cells into chondrocytes which in turn secrete extracellular matrix components to form cartilage at the site of the defect *in situ* (e.g. U.S. Pat. Nos. 5,206,023 and 5,270,300 and EP-A-530,804). This approach however results in problems similar to those associated with the first approach hereinabove. Furthermore there is no data so far that articular cartilage contains chondrocytic progenitors for partial-thickness defects.

In the third approach, chondrocytes may be cultured and expanded *in vitro* to form synthetic cartilage-like material that is implanted subsequently into the cartilage defect. This has the advantage over the previous methods in that the development of the synthetic cartilage material may be monitored, through biochemical and

morphological characterization, prior to implantation. Growing chondrogenic cells may be achieved in either an anchorage-dependent or an anchorage-independent manner. In the latter, chondrogenic cells may be cultured as colonies within an agarose gel. Heretofore, only small pieces of cartilage tissue of undefined shape have been prepared using this manner. Furthermore, the resulting cartilage remains embedded within a gel matrix making it less suitable for implantation into mammals. Alternatively, in another anchorage-independent method, chondrocytes may be cultured as colonies in suspension culture. However the resulting particles containing synthetic cartilage-like material are usually small and of undefined shape thus making them unsuitable for implantation and repair of a predetermined articular cartilage defect. This would rather result in several little pieces of cartilage, completely separated from each other, and far from being very well integrated among them and the surrounding cartilaginous tissue.

In the anchorage-dependent method, primary cultures of chondrocytes isolated from primary tissue are grown as monolayers attached to the surface of a cell culture flask (e.g. U.S. Pat. No. 4,356,261). The primary cells derived directly from explant tissue remain capable of producing and secreting extracellular components characteristic of natural cartilage, specifically type II collagen and sulfated proteoglycans. However, it is well known that during *in vitro* expansion procedures chondrocytes in monolayer undergo a dedifferentiation process, thereby losing their ability to organize hyaline cartilage *in vivo*. Therefore, until now it has not been possible to prepare large patches of articular cartilage from small pieces of biopsy tissue using the anchorage-dependent procedures of U.S. Pat. No. 4,356,261.

In order to solve the above problems, U.S. Pat. No. 5,723,331 provides a method for preparing *in vitro* large quantities of synthetic cartilage from small samples of biopsy tissue which, based on the discovery that chondrogenic cells may be isolated from a variety of tissues, e.g. pre-existing cartilage, perichondrial tissue or bone marrow, and expanded *in vitro* prior to cartilage formation, includes first seeding denuded (i.e. isolated from an enzymatically or mechanically disaggregated tissue) chondrogenic cells, proliferated *ex vivo*, into a pre-shaped well having a cell contacting, cell adhesive surface, and then culturing the proliferated chondrogenic cells in the well for a time sufficient to permit the cells to secrete an extracellular matrix thereby to form a three-dimensional, multi cell-layered patch of synthetic cartilage.

A further disadvantage of these methods is that the chondrocytes must be obtained from the patient, typically by a biopsy, culture expanded, and then implanted on a matrix. This is relatively easy in laboratory animals, but presents greater logistical problems in humans where a defect is created by the biopsy required to provide cells for

repair of another defect. Moreover, if the defect includes a part of the underlying bone, this is not corrected using chondrocytes, which are already differentiated and will not form new bone. The bone is required to support the new cartilage.

The use of mesenchymal stem cells has also been proposed for the repair of many tissues including cartilage. Mesenchymal stem cells are a potential alternative source of cartilage-producing cells. They are generally recognized as pluripotent cells which are capable of dividing many times to produce progeny cells that can eventually give rise to connective tissues, including cartilage, bone, tendons, ligaments, marrow stroma. By definition, they are not limited to a fixed number of mitotic divisions.

Stem cells are defined as cells that are undifferentiated, which can divide without limit to yield cells that are either stem cells or cells that further differentiate to yield different types of progenitor cells, including mesenchymal stem cells. Those mesenchymal stem cells are pluripotential cells that are capable of differentiating into any of the specific types of mesenchymal or connective tissues, including skeletal tissues. Mesenchymal stem cells were isolated from bone marrow or other sources such as periosteum, placenta, umbilical cord, skin, and blood (e.g. in U.S. Pat. No. 5,811,094). Pluripotent mesenchymal stem cells have also been isolated from muscle (Patè et al., *Proc. 49th Ann. Sess. Forum Fundamental Surg. Problems* Oct. 1993, 587-9), heart (Dalton et al., *J. Cell Biol.* 1993, 119 R202) and granulation tissue (Lucas et al., *J. Cell. Biochem.* 1993, 122 R212). Pluripotency was demonstrated using a non-specific inducer, dexamethasone, which elicits differentiation of the stem cells into chondrocytes (cartilage), osteoblasts (bone), myotubes (muscle), adipocytes (fat), and connective tissue cells.

Unfortunately, although it is highly desirable to have stem cells which are easily obtained by a muscle or a skin biopsy, cultured to yield large numbers, and then used as a source of chondrocytes or osteoblasts or myocytes, there is no known specific inducer of the mesenchymal stem cells that yields only cartilage. *In vitro* studies in which differentiation is achieved yield a mixture of cell types. In U.S. Pat. Nos. 5,226,914 and 5,197,985 the cells were seeded into porous ceramic blocks and, subcutaneously implanted into nude mice, yielded primarily bone. However, U.S.Pat. No. 5,906,934 discloses that under very specific conditions mesenchymal stem cells in a suitable polymeric carrier (such as polyglycolic acid mesh) implanted into a cartilage and/or bone defect will differentiate to form cartilage and/or bone, as appropriate. Also U.S. Pat. No. 5,919,702 discloses chondrocyte progenitor cells isolated from umbilical cord sources, e.g. from Wharton's jelly, and cultured so as to give rise to chondrocytes that can produce cartilage tissue. Also in another attempt to avoid the drawbacks of current

cartilage and bone repair techniques which cause bleeding and involve the use of mechanically weak non self-derived material, U.S. Pat. No. 5,866,415 suggests treating cartilage or bone defects with a biological material obtained by attaching *in vitro* cartilage or bone forming cells to a periosteum of sufficient size to accomodate the defect.

WO/96/41523 and WO96/41620 describe the use of FGFR3 as a marker for mesenchymal skeletal progenitor cells. Such cells, however, express FGFR3 which has been determined by the present inventors to indicate differentiation into non-pluripotent cells of the prechondroblast type in humans. Hence, the cells selected by these known methods differ from the precursor cells selected in accordance with the present invention.

Figure 1 shows schematically the hierarchical cascade of cells in the differentiation process, starting from the undifferentiated mesenchymal stem cells downwards to the fully differentiated cells of the skeleton. U.S. Pat. No. 5,811,094 describes methods to identify, selectively isolate and enrich by culture expansion mesenchymal stem cells. Said patent does not provide methods for isolating, purifying, and culturally expanding skeletal precursor cells, methods which are the purpose of the present invention. Our efforts are focused on the skeletal precursor cells, as hereinafter defined, unraveling the molecular cascade of events underlying the differentiation pathways leading to the specialized cells of the skeletal tissues, with specific attention to the generation of the stable chondrocytes. Stable chondrocytes are assumed to form stable cartilage *in vivo* and/or *in vitro* under appropriate conditions. With stable cartilage is meant that any signs of bone formation remain absent, even over longer periods.

Transforming growth factor-.beta ("TGF-β") refers to a family of related dimeric proteins, which regulate the growth, and differentiation of many cell types. Members of this family include TGF-β 1, TGF-β 2, TGF-β 3, TGF-β 4, TGF-β 5, morphogenic proteins ("MP") such as MP-121 and MP-52, inhibins/activins (such as disclosed in EP-A-222,491), osteogenic proteins ("OP"), bone morphogenetic proteins (hereinafter denoted "BMP"), growth/differentiation factors ("GDF") such as GDF-5, GDF-6, GDF-9 and Nodal. TGF-β was first characterized for its effects on cell proliferation. It both stimulated the anchorage-independent growth of rat kidney fibroblasts and inhibited the growth of monkey kidney cells. TGF-β family members have been shown to have many diverse biological effects, e.g. they regulate bone formation, induce rat muscle cells to produce cartilage-specific macromolecules, inhibit the growth of early hematopoietic progenitor cells, T cells, B cells, mouse keratinocytes, and several human cancer cell lines. TGF-β family members increase the synthesis and secretion of collagen and fibronectin,

accelerate healing of incisional wounds, suppress casein synthesis in mouse mammary explants, inhibits DNA synthesis in rat liver epithelial cells, stimulate the production of BFGF binding proteoglycans, modulate phosphorylation of the epidermal growth factor ("EGF") receptor and proliferation of epidermoid carcinoma cells and can lead to apoptosis in uterine epithelial cells, cultured hepatocytes and regressing liver. TGF-βs can mediate cardio-protection against reperfusion injury by inhibiting neutrophil adherence to endothelium and it protects against experimental autoimmune diseases in mice. On the whole, proteins of the TGF-β family are multifunctional, hormonally active growth factors and also have related biological activities such as chemotactic attraction of cells, promotion of cell differentiation and tissue-inducing capabilities. Differences in their structure and their affinity for receptors lead to considerable variations in their exact biological function.

In contrast to the foregoing reports of the ability of TGF-β to induce the production of cartilage-specific macromolecules in muscle cells and chondrocytes, TGF-β was found to act synergistically with fibroblast growth factor to inhibit the synthesis of collagen type II by chicken sternal chondrocytes and in rat chondrocytes. In fact, TGF-β has emerged as the prototypical inhibitor of the proliferation of most normal cell types *in vitro* as well as *in vivo*, exhibiting a remarkable diversity of biological activity. TGF-β 1 has been purified from human and porcine blood platelets and recombinant TGF-β 1 is currently available.

Among the sub-family of BMPs, the structures of BMP-1 through BMP-13 have previously been elucidated. The unique inductive activities of these proteins, along with their presence in bone, suggests that they are important regulators of bone repair processes and may be involved in the normal maintenance of bone tissue. Recently, the BMP-12-related subfamily of proteins, including BMP-13 and MP52 (see e.g. WO93/16099 and U.S. Pat. No. 5,658,882), was shown to be useful in compositions for the induction of tendon/ligament-like tissue formation and repair. U.S. Pat. No. 5,902,785 discloses that BMP-12 related proteins are particularly effective for the induction of cartilaginous tissue and that BMP-9 is useful for increasing proteoglycan matrix synthesis and therefore for the maintenance of cartilaginous tissue. It also describes compositions comprising a BMP-12 related protein and additionally including one or more TGF-β proteins proven to be osteogenic, preferably BMP-2, -4, -5, -6 and/or BMP-7 as useful for the regeneration of multiple tissue types (for example at the interface or junction between tissues) and especially useful for the treatment of articular cartilage, in which the articular surface, cartilage, subchondral bone and/or tidemark interface between cartilage and bone need to be repaired. The same patent further

describes compositions comprising a BMP-12 related protein together with a protein useful for the maintenance of chondrocytes or cartilaginous tissue such as BMP-9, the said compositions being especially useful for the induction and maintenance of cartilaginous tissue at a site in need of cartilage repair such as an articular cartilage defect.

WO96/14335 discloses, using mRNA prepared from newborn articular cartilage, the isolation of two members of the BMP family, designated Cartilage-derived morphogenetic proteins-1 and -2 (CDMP-1, CDMP-2). More specifically, WO96/14335 discloses a purified cartilage extract that stimulates local cartilage formation when combined with a matrix and implanted into a mammal, said extract being produced by obtaining cartilage tissue, homogenizing said cartilage tissue in the presence of chaotropic agents under conditions that permit separation of proteins from proteoglycans, separating said proteins from said proteoglycans, and obtaining said proteins. It also discloses an isolated DNA molecule encoding a protein having chondrogenic activity *in vivo* but substantially no osteogenic activity *in vivo*. The role of CDMP-1 as a regulator of skeletal growth is now well documented. Storm et al. (1994) in *Nature* 368, 639-43 and Chang et al. (1994) in *J.Biol.Chem.* 269, 28227-34 independently established that CDMP-1 mapped close to the brachypodism locus on chromosome 2 in mice and might be involved in the brachypodism phenotype. Also the expression patterns of CDMPs suggests an important role for these genes in joint morphogenesis. WO98/59035 also discloses a method of maintaining a cartilaginous phenotype in chondrocytes *in vitro*, comprising culturing the chondrocytes in a serum-free medium containing such a CDMP and/or BMP. Table 1 below summarizes the BMP superfamily members in mammals (Reddi AH, *Nature Biotechnol.* 1998, 16: 247-52).

Table 1

BMP subfamily	Generic name	BMP designation
BMP 2/4	BMP-2A BMP-2B	BMP-2 BMP-4
BMP 3	Osteogenin Growth/differentiation factor 10	BMP-3 BMP-2B
Op-1/BMP-7	BMP-5 Vegetal related-1 (Vgr-1) Osteogenic Protein-1 (Op-1)	BMP-5 BMP-6 BMP-7

	Osteogenic Protein-2 (Op-2)	BMP-8
	Osteogenic Protein-3 (Op-3)	BMP-8B
	Growth/differentiation factor 2 (GDF-2)	BMP-9
	BMP-10	BMP-10
	Growth/differentiation factor 11 (GDF-11)	BMP-11
GDF-5,6,7	Growth/differentiation factor 7 (GDF-7) or cartilage-derived morphogenetic protein-3 (CDMP-3)	BMP-12
	Growth/differentiation factor 6 (GDF-6) or cartilage-derived morphogenetic protein-2 (CDMP-2)	BMP-13
	Growth/differentiation factor 5 (GDF-5) or cartilage-derived morphogenetic protein-1 (CDMP-1)	BMP-14
	BMP-15	BMP-15

Other families of growth factors have been shown to be involved in cartilage differentiation and maintenance such as the fibroblast growth factors (FGFs), which are a family of polypeptide growth factors involved in a variety of activities. One of their receptors, FGF receptor 3 (FGFR-3) (Keegan K. et al., *1991* Proc. Nat. Acad. Sci. 88: 1095-99), is known to play a crucial role in chondrogenesis. Point mutations in the fgfr3 gene resulting in a ligand-independent constitutively active protein (which means that the FGF signaling is always active also in the absence of the ligand) cause skeletal abnormalities as achondroplasia and thanatophoric dysplasia.

As already outlined in page 2, although autologous chondrocyte transplantation ("ACT") is becoming a widely accepted technique for repair of joint surface defects ("JSD"), it still presents some drawbacks. More in detail, this procedure implies *in vitro* expansion - in the presence of autologous serum - of autologous chondrocytes obtained from an uninvolved area of the joint, followed by the implantation of the chondrocyte suspension under a periosteal flap sutured to seal the joint surface defect. Cell expansion is necessary to obtain from a small cartilage biopsy a number of cells sufficient to repair the cartilage defect. However, it is well known, as explained before, that *in vitro* expansion of chondrocytes results in cell de-differentiation. This implies that chondrocyte expansion pays the price of loss of phenotypic stability. Therefore, a quality control for expanded chondrocytes to be used for ACT is needed. At the end of cell expansion the chondrocyte population is composed of some cells that retain their

phenotypic stability, and others that still can proliferate but will not anymore contribute to cartilage repair. In order to obtain a consistent cell suspension for ACT, it is desirable to select stable chondrocytes within the expanded cell population. Chondrocytes are skeletal cells able to grow in anchorage-independent agarose cultures. The ability of chondrocytes to grow in anchorage-independent conditions is critical for those cells to survive and organize cartilage tissue once injected as a cell suspension for repair of JSD, but is probably not the only phenotypic trait required.

Therefore there is a need in the art for identifying and selecting an easily accessible and expandable source of pluripotent skeletal precursor cells. There is a need in the art for solving the various problems encountered in the cartilage repair known methods. There is also a need in the art for developing repair techniques for connective tissues including cartilage, e.g. for medical problems such as rheumatoid arthritis and osteoarthritis, and a long felt need for quality control on the chondrocyte populations used for such purposes. There are a number of suggestions in the prior art that some mesenchymal stem cells could specifically yield cartilage or, as needed, other connective tissues. For instance bone marrow contains populations of pluripotent mesenchymal stem cells having the capacity to differentiate into a wide range of cell types of the mesenchymal, hematopoietic and stromal lineages. It is also known that mesenchymal stem cells cultured *in vitro* can be induced to differentiate into bone or cartilage *in vivo* and *in vitro*, depending upon the tissue environment or the culture medium into which the cells are placed. To date, however, very few common cell markers or differentiation antigens were identified. Examples of such markers include Ly-6 antigens for murine osteoblasts (see Horowitz et al. (1994) in *Endocrinology*, 135, 1032-43) and CD34 for human hematopoietic cell types. On the other hand, periosteum and marrow are known as the most common sources of precursor cells having osteogenic potential. More specifically it has been shown that cells from marrow, when isolated and expanded by the culture method of Friedenstein, will form bone, cartilage and fibrous tissue when implanted. However Friedenstein in *Calcif.Tiss.Int.* (1995) 56(S):S 17 admitted that obstacles, such as the need for culturing for several passages and developing a method for transplanting such cells, must be overcome before clinical utility of this discovery can be confirmed. Therefore there is a need in the art for the proper identification of pluripotent skeletal precursor cells in a wide range of easily accessible and expandable sources. These goals and other purposes are achieved by means of the following objects of the present invention.

SUMMARY OF THE INVENTION

A first object of the present invention is the identification and characterization of skeletal precursor cells in a wide range of easily accessible and expandable sources. Easily accessible tissues include among others, periosteum, bone marrow and synovial membrane. The solution to this problem in accordance with the present invention is to use a set of molecular markers. These molecular markers may be either positive markers, indicating precursor cells which are pluripotent or negative markers indicating that the cells have differentiated and are no longer pluripotent. Absence of a negative marker can be used as a positive marker. A second object of the present invention is the use of such skeletal precursor cells and molecular markers for the repair of a wide range of connective tissues. A third object of the present invention is the use of such skeletal precursor cells as a source of transforming growth factors ("TGF") linked to the phenotypic stability of a certain cell population involved in a certain differentiation pathway, such as for instance members of the TGF-β family which are positively associated with chondrocyte phenotypic stability. A fourth object of the present invention is the use of such skeletal precursor cells and molecular markers as matrix producing cells in tissue engineering procedures. A fifth object of the present invention is the co-implantation of expanded skeletal precursor cells and chondrocytes for *in vivo* cartilage repair.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the hierarchical cascade of cells in the differentiation pathways.

Figure 2 is a picture showing that skeletal precursor cells express CDMP-1.

Figure 3 shows how the phenotype of skeletal precursor cells does not depend on cell passage number or donor age. Periosteum-derived cells at P5 and P15 from four donors of different ages have a comparable molecular profile as assessed by semi-quantitative RT-PCR.

Figure 4 consists of two histological pictures showing that skeletal precursor cells can grow anchorage-independently retaining their phenotypic stability *in vivo*.

Figure 5 is a picture showing that the implant retrieved from mouse muscle is formed by human cells.

Figure 6 consists of pictures demonstrating that chondrogenic differentiation is obtained independent of the age of the donors.

Figure 7 shows histological pictures of cartilaginous tissue obtained *in vitro* from human skeletal precursor cells.

Figure 8 A-F shows histochemical and immunohistochemical analysis of

micromasses either untreated (A, B, H) or treated with TGF-β1.

Figure 9 shows the gene expression dynamics by RT-PCR during chodrogenesis in CDMP-1 marked skeletal precusor cells.

DETAILED DESCRIPTION OF THE INVENTION

Terms used throughout this disclosure are defined as follows:

Chondrocyte stability

The capacity of a cell suspension (either obtained from cartilage tissue or from any other tissue containing cells with chondrogenic potential) to produce *in vitro* and/or upon injection in a mammal (*in vivo*), such as immune-deficient mice, in a time frame of 2-3 weeks a true-to-type (hyaline) cartilage implant without signs of vascular invasion or endochondral bone formation

Chondrogenic

The capacity to promote or stimulate cartilage growth, as applied to chondrocytes and to cells which themselves differentiate into chondrocytes. The term also applies to certain growth factors, such as TGF-β, which promote cartilage growth.

Co-expression

With co-expression, in the context of the present invention, is meant that a factor is expressed whenever another factor or marker is expressed in or on a cell. For instance, where a morphogenic protein is used as a marker, and more in particular the cartilage-derived morphogenic protein CDMP-1 or a homolog thereof are/is expressed, co-expression requires that a co-expressed marker is only present or expressed when the morphogenic marker is expressed. Hence, the factor is linked with the same specific post-natal differentiation pathway as the morphogenic protein it co-expresses with, such as CDMP-1. It preferentially is upregulated/downregulated together with the marker. It will for instance downregulate when the precursor cells undergo differentiation such as towards the chondrocytic phenotype. Such co-expressing marker further is preferably expressed at detectable levels. Such co-expressed factor can be a recognizable cell surface marker, detectable via polyclonal or monoclonal antibodies and/or specific ligands. The co-expressed factor may also include any functional or structural homolog of CDMP-1.

Connective tissue

As used herein, any of a number of structural tissues in the body of a mammal including bone, cartilage, ligament, tendon, meniscus, muscle, dermis, hyperdermis and joint capsule.

Differentiation